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Solution Stereochemistry of Cyclophosphamide and Rigid Model Analogues

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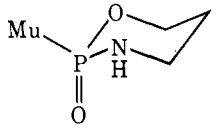
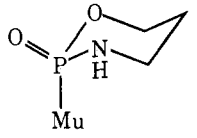
Received September 11, 1978

Abstract: The structural properties of the anticancer drug cyclophosphamide (**1**, Mu = $(ClCH_2CH_2)_2N$) in solution are clarified by comparison of its spectroscopic characteristics with those of the rigid *cis*-4,6-dimethyl analogues **2a** and **2b**. The latter compounds, along with the *trans*-4,6-dimethyl isomers **3a** and **3b** (in which Mu is either axial or equatorial), were synthesized by ammonolysis of cyclic sulfate esters of *dl*- and *meso*-HOCHMeCHMeOH (**5a** and **5b**) followed by hydrolysis to the threo and erythro amino alcohols, respectively, and ring closure with $MuP(O)Cl_2$. In this synthetic scheme, isomerization of **5b** to **5a** was observed in acid and inversion at carbon apparently takes place in the ammonolysis step. ^1H NMR evidence is presented for the stereochemistries shown for the methyl groups in the principal conformers of **2a,b**, **3a,b**, and **5a,b**. Isomerism at phosphorus in **2a** and **2b** is indicated by examination of the N–H stretching region in the IR as well as by comparison of ^{31}P and ^1H NMR parameters. Detailed LIS investigations yield low *R* factors at high confidence levels for the structures shown. Similar experiments with **1** reveal a tendency toward conformation **1a** which is stronger in the presence of an LIS reagent but weaker in the presence of a hydrogen-bonding solvent such as water or chloroform. Although the stereochemistry of the methyl groups in **3a** and **3b** can be deduced from ^1H NMR data, assignment of the phosphorus stereochemistries in their dominant conformers is somewhat ambiguous. Preliminary antitumor cell screening indicates that **2a** is more active against KB cell cultures than **2b**.

As one of the most widely used chemotherapeutic agents in the treatment of many types of cancer, much effort has been made to understand the mode of action of cyclophosphamide (**1**) and to develop analogues with improved action.[1] Reports



1a



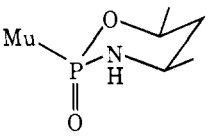
1b

Mu = $(ClCH_2CH_2)_2N$

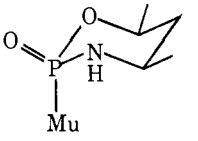
of structural investigations of cyclophosphamide and its ring carbon substituted analogues have been almost totally confined to solid-state X-ray investigations which reveal the presence of conformation **1a**.[2] Recently cyclophosphamide has been resolved[3] and the (+) enantiomer, which is more readily metabolized in human patients,[4] has been shown to have the *R* absolute configuration[5] while the (−) enantiomer, which is more active against PC6 mouse tumors,[6] has been demonstrated to possess the *S* configuration.[7]

In this paper we address ourselves to the stereochemical nature of **1** in solution, the state in which it displays its biological action. To obtain spectroscopic information characteristic of conformers **1a** and **1b**, we have synthesized the isomeric *cis*-4,6-dimethyl analogues **2a** and **2b**. Also reported are the isomeric *trans*-4,6-dimethyl compounds **3a** and **3b**.

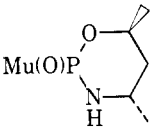
Previous efforts to elucidate the solution behavior of **1** have been few. Data from variable-temperature ^{13}C and ^1H NMR studies of **1** are consistent with a low barrier to ring reversal in the equilibrium **1a** ⇄ **1b**.[8] Attempts to elucidate the solution configurations of the isomeric 4-methyl derivatives of cyclophosphamide **4a** and **4b** by NMR and IR spectroscopic means have led to conflicting structural assignments.[9,10]



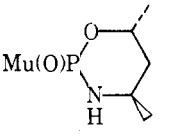
2a



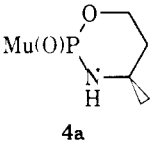
2b



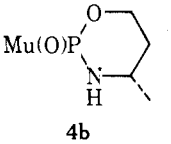
3a



3b



4a



4b

Experimental Section

Materials. Solvents and reactants, unless specifically noted otherwise, were reagent grade or better. Aromatic solvents were dried with NaK alloy, 4A molecular sieves, or KOH pellets, THF with K_2CO_3 or $LiAlH_4$, triethylamine with KOH pellets, ether with Na or Na/K alloy, and 1,2-dichloroethane, acetone, hexanes, carbon tetrachloride, and ethyl acetate with 4A molecular sieves. Ethanol was removed from preserved chloroform by washing several times with half a volume of water per volume of the solvent followed by drying for at least 1 day over anhydrous calcium chloride and distilling onto magnesium sulfate or 4A molecular sieves. Phosphoryl trichloride was distilled be-

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fore use. Tris(2,2,6,6-tetramethyl-3,5-heptanedionato)europium ($Eu(dpm)_3$) was purchased from Aldrich Chemical Co., Inc.

Spectral Measurements. All ^1H NMR spectra were obtained with a Varian A-60 or HA-100 or Bruker HX-90 spectrometer on solutions containing tetramethylsilane as internal standard. ^{13}C and ^{31}P NMR spectra were obtained with a Bruker HX-90 spectrometer operating at 22.63 and 36.43 MHz, respectively, in the FT mode employing gated or white-noise proton decoupling and locked on deuterium of the deuterated solvent. Tetramethylsilane and 85% H_3PO_4 were used as internal and external standards for the ^{13}C and ^{31}P NMR spectra, respectively, with downfield shifts considered positive. All but the C(4) CH_3 and C(6) CH_3 ^{13}C chemical shift assignments of **2a,b** and **3a,b** were made on the basis of the splitting patterns arising from proton coupling and relative peak areas. The C(4) CH_3 and C(6) CH_3 chemical shifts were arrived at by comparison of these resonances with the assignment of the C(4) CH_3 absorptions in **4a,b**.[9] Varying degrees of certainty surround the assignment of the mustard carbon ^{13}C resonances in **1**[9,10] and **4a,b**.[10] Because assignment is ambiguous we do not differentiate between these carbons in our assignments. A Beckman 4250 spectrometer was used to obtain infrared spectra which were calibrated with polystyrene.

Mass spectra were obtained with an AEI MS-902 high-resolution spectrometer. Exact masses were determined by peak matching with standards.

Other Measurements. Melting points were obtained in open capillaries on a Thomas Uni-Melt apparatus and are uncorrected. The dipole moment instrumentation, procedures, and data treatment have been described in detail elsewhere.[11] Three to five independent solutions of each compound ranging in concentration from about 1×10^{-3} to 10×10^{-3} mole fraction in benzene solution at 25.0 °C were employed. Lanthanide-induced shifts were obtained by adding $Eu(dpm)_3$ in increments to approximately 0.2 M solutions of each compound in CCl_4 with chemical shifts of protons being measured relative to internal Me_4Si.

Syntheses. Anhydrous cyclophosphamide was prepared by following a previously described procedure.[3b] Synthesis of an isomeric mixture of 2,4-pentanediols was also made according to a reported method.[12]

***cis*- and *trans*-4,6-Dimethyl-2,2-dioxo-1,3,2-dioxathiacycloheptanes (5a, 5b).** Up to 7 mol of chlorosulfonic acid (Baker Practical Grade) in 1–3 volumes of ethanol-free chloroform or 1,2-dichloroethane was added dropwise to a solution of one-half as many moles of an approximately 1:1 isomer mixture of 2,4-pentanediols in 3–4 volumes of the same solvent which was well stirred, ice-water cooled, and continuously purged by prepurified nitrogen. During the addition, hydrogen chloride was evolved and a dense, oily, dark red phase separated from a much larger yellow to red lighter phase. Stirring and gas purging at room temperature were continued for 1–3 h. The two liquid layers were then separated and the denser red liquid was extracted several times with equal volumes of the solvent used in the reaction. The combined lighter layer and washings were carefully neutralized with concentrated aqueous ammonia, washed several times with distilled water, and dried overnight with sodium or magnesium sulfate. Concentration of the dried organic layer gave a pale yellow liquid containing both cis and trans cyclic sulfates in at least 50% yield. The ratio of cis:trans sulfates always exceeded the ratio of *meso*-:*d,l*-2,4-pentanediols in the starting material and this excess increased as the time between reaction and workup was lengthened.

Some of the cis isomer could be crystallized from the product mixture by cooling an alcohol, ether, or chloroform solution (mp 40.0–41.5 °C, lit.[13] 40 °C; ^1H NMR ($CDCl_3$) δ 1.48 d, $^3J_{HH}$ = 6.3 Hz, 6 H, CH_3, 1.85 m, $^2J_{HH}$ = 14.4, $^3J_{HH}$ = 12.1 Hz, 1 H, CHH_{ax}, 2.03 m, $^2J_{HH}$ = 14.4, $^3J_{HH}$ = 2.1 Hz, 1 H, CHH_{eq}, 5.07 m, $^3J_{HH}$ = 2.1 and 12.1 Hz, 2 H, $MeCH_{ax}$). A partial separation of the remaining cis–trans mixture was achieved by chromatography on 60/200 mesh silica with benzene. Thus the first few fractions from the column are almost pure cis while the last few are nearly pure trans (^1H NMR (C_6H_6) δ 1.19 d, $^3J_{HH}$ = 6.6 Hz, 6 H, CH_3, 1.42 t, $^3J_{HH}$ = 5.62 Hz, 2 H, CH_2, 4.72 ca. 1:5:10:10:5:1 m with further splitting of inner four peaks, 2 H, CH_3CH). Both isomers have very similar mass spectra. Although no parent ions could be discerned at 70 or 18 eV, a fragment of *m/e* 151 corresponding to $P - Me^+$ was the base peak for each compound. Since the next step in the synthesis did not require separation of the isomers, no further efforts to purify them were made.

***threo*- and *erythro*-4-Amino-2-pentanol (6a, 6b).** Although the preparation,[14a,14b] separation,[14a] and partial separation[14b] of these amino alcohols have been described, a synthetic method patterned after another procedure[15] for a related amino alcohol was found to be quite convenient. Both the cis as well as an isomeric mixture of 2,4-pentanediol cyclic sulfates **5a,b** were carried through the following steps which are described for a mixture of the sulfates.

The cyclic sulfates were stirred at room temperature with 3–5 volumes of concentrated aqueous ammonia until essentially no second liquid phase was present. Since the trans sulfate was found to react more rapidly than the cis isomer, a partial separation could be made if desired. It was possible to realize enrichment of the product from the trans sulfate in the aqueous layer by interrupting the reaction and separating the layers. Upon completion of the reaction, the solution was concentrated to a viscous oil or moist, white mass under reduced pressure.

The crude product from the ammonolysis was heated with a large excess of 8 N HCl at 100–125 °C for several hours or until a ^1H NMR spectrum showed that the starting material had disappeared. The cooled reaction mixture was washed with ether to remove a small, brown, oily phase and then concentrated to a small volume.

The concentrate was treated with concentrated aqueous sodium hydroxide and sodium hydroxide beads until the pH exceeded 11 and a second liquid layer separated. The solid/liquids mixture was filtered, the liquid layers were separated, and all phases were extracted several times with chloroform. The combined chloroform extracts were dried with K_2CO_3 and concentrated. The 4-amino-2-pentanol was purified by reduced-pressure distillation (yield ca. 50% based on cyclic sulfates) and separated by a second distillation with a Teflon spinning-band fractionation column to give *threo*-4-amino-2-pentanol (bp 73–74 °C (15–16 Torr), lit.[14] 60 °C (12 Torr); parent ion *m/e* calcd 103.099 72, found 103.099 78; ^1H NMR ($CDCl_3$) δ 1.16 d, $^3J_{HH}$ = 6.6 Hz, 3 H, CH_3, 1.19 d, $^3J_{HH}$ = 6.4 Hz, 3 H, CH_3, 1.37–1.59 m, 2 H, CH_2, 2.96 s, 3 H, OH, NH_2, 3.10–3.64 m, 1 H, H_2NCH, 3.86–4.38 m, 1 H, $HOCH$) and *erythro*-4-amino-2-pentanol (bp 67–69 °C (15–16 Torr), lit.[14] 61–63 °C (12 Torr); parent ion *m/e* found 103.099 99; ^1H NMR ($CDCl_3$) δ 1.13 d, $^3J_{HH}$ = 6.2 Hz, 6 H, CH_3, 1.30–1.50 m, 2 H, CH_2, ca. 2.9 br singlet, 3 H, OH, NH_2, 2.78–3.32 m, 1 H, H_2NCH, 3.72–4.24 m, 1 H, $HOCH$).

Bis(2-chloroethyl)aminophosphoryl Dichloride. Although this compound has been reported previously,[16] the following procedure was found convenient. Two moles of triethylamine in 350 mL of toluene was added dropwise to a stirred suspension of 1 mol of bis(2-chloroethyl)amine hydrochloride in 1 mol of phosphoryl trichloride and 350 mL of toluene in a nitrogen atmosphere. After addition, the mixture was heated to reflux for several hours, cooled, and filtered. The product was purified by distillation (bp 126–129 °C (1.5 Torr) or 106–107 °C (0.4–0.5 Torr), lit.[16] 123–125 °C (0.6 Torr); 75% yield) or by recrystallization from acetone–hexanes.

2-[Bis(2-chloroethyl)amino]-2-oxo-*cis*-4,6-dimethyl-1,3,2-oxazaphosphorinane (2a, 2b). Bis(2-chloroethyl)aminophosphoryl dichloride (0.0417 mol in 50 mL of THF) was added dropwise to stirred *erythro*-4-amino-2-pentanol (0.0417 mol) and triethylamine (0.0834 mol) in 125 mL of THF under a nitrogen atmosphere. After addition, the reaction mixture was stirred for at least 5 h and then filtered. Reduced-pressure distillation of the volatiles left a very viscous, colorless liquid (^{31}P NMR ($CDCl_3$) δ 10.2 and 12.0 in area ratio of 1.14:1).

Some of the less soluble isomers could be crystallized from chloroform–hexanes, benzene–hexanes, or ethyl acetate–hexanes. The resulting mother liquor was partially separated on 60/200 mesh "Baker Analyzed" silica gel with 195:5 chloroform–methanol. The fractions containing at least 80% of the more mobile isomer (which was also the more soluble isomer in crystallization solvents) were combined and the more mobile isomer crystallized from the same liquid mixtures used for the less mobile isomer. This cycle (crystallization of the slower isomer, chromatography, crystallization of the faster isomer) was applied to the remaining chromatography fractions. The slow isomer **2a** was realized in about 50% yield (mp 91.0–93.5 °C; ^1H NMR ($CDCl_3$) δ 1.18 dd, $^3J_{HH}$ = 6.4, $^4J_{PH}$ = 2.8 Hz, 3 H, $NCCH_3$, 1.33 dd, $^3J_{HH}$ = 6.4, $^4J_{PH}$ = 2.1 Hz, $OCCH_3$, 1.54–1.99 m, 2 H, CCH_2C, 3.09 s, 1 H, NH, 3.19–3.88 m, 9 H, $ClCH_2CH_2$, $NCHCH_3$, 4.33–4.86 m, 1 H, OCH; ^{31}P NMR ($CDCl_3$) δ 12.9; ^{13}C NMR ($CDCl_3$) δ 73.8 d, $^2J_{PC}$ = 5.9 Hz, 1 C, C(6), 41.0, 1 C, C(5), 47.6, 1 C, C(4), 24.0, $^3J_{PC}$ = 12.5 Hz, 1 C, C(4) CH_3, 22.5, $^3J_{PC}$ = 8.8 Hz, 1 C, C(6) CH_3, 48.9 $^{2or3}J_{PC}$ = 4.4 Hz, 1 C, C(Mu), 42.3, 1 C, C(Mu)). The fast isomer **2b** was obtained in approximately 40% yield (mp 70.5–72.5 °C; ^1H NMR ($CDCl_3$) δ 1.23 dd, $^3J_{HH}$ = 6.3,

$^4J_{PH}$ = 2.5 Hz, 3 H, $NCCH_3$, 1.36 dd, $^3J_{HH}$ = 6.3, $^4J_{PH}$ = 2.0 Hz, 3 H, $OCCH_3$, 1.54–1.91 m, 2 H, CCH_2C, 2.99–3.86 m, 10 H, $ClCH_2CH_2$, NH, $NCHCH_3$, 4.11–4.66 m, 1 H, OCH; ^{31}P NMR ($CDCl_3$) δ 10.2; ^{13}C NMR ($CDCl_3$) δ 75.8, $^2J_{PC}$ = 8 Hz, 1 C, C(6), 41.6, 1 C, C(5), 48.0, 1 C, C(4), ca. δ 23.0, 2 C, C(6) CH_3, C(4) CH_3 with uncertain $^3J_{PC}$ due to peak overlaps, 48.6, $^{2or3}J_{PC}$ = 3.6 Hz, 1 C, C(Mu), 42.0, $^{2or3}J_{PC}$ = 3.6 Hz, 1 C, C(Mu)).

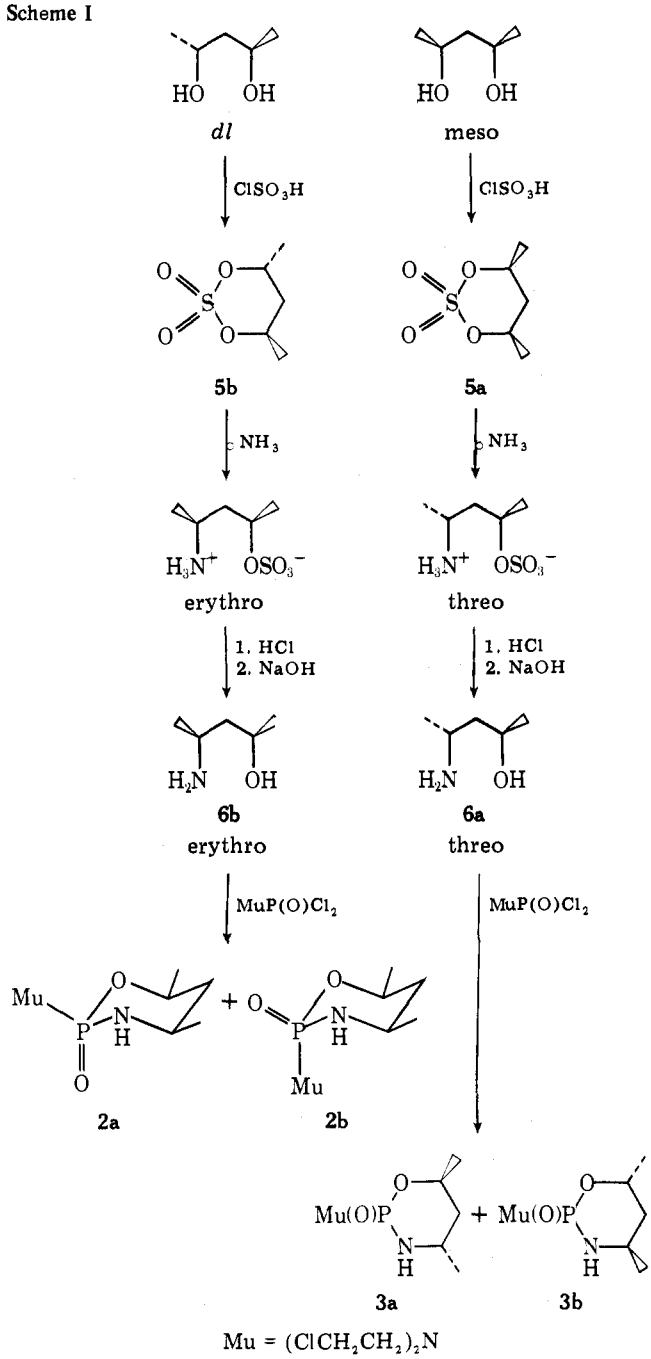
2-[Bis(2-chloroethyl)amino]-2-oxo-*trans*-4,6-dimethyl-1,3,2-oxazaphosphorinanes (3a, 3b). The synthetic and separation methods given for the *cis*-dimethyl analogues **2a** and **2b** were followed. The crude reaction product gave ^{31}P resonances at 10.2 and 10.9 ppm ($CDCl_3$) in a 1.44:1 area ratio. The chromatographically slower isomer **3a** was realized in about 60% yield (mp 83.5–84.5 °C; parent ion *m/e* calcd 288.056 13, found 288.058 03; ^1H NMR ($CDCl_3$) δ 1.19 dd, $^3J_{HH}$ = 6.4, $^4J_{PH}$ = 2.4 Hz, 3 H, $NCCH_3$, 1.47 d, $^3J_{HH}$ = 6.8 Hz, 3 H, $OCCH_3$, 1.60–1.82 m, 2 H, CCH_2C, 3.14–3.77 m, 10 H, $ClCH_2CH_2$, NH, $NCHCH_3$, 4.17–4.97 m, 1 H, OCH; ^{31}P NMR ($CDCl_3$) δ 10.2; ^{13}C NMR ($CDCl_3$) δ 73.8, $^2J_{PC}$ = 7.3 Hz, 1 C, C(6), 37.8, $^3J_{PC}$ = 5.9 Hz, 1 C, C(5), 43.7, $^2J_{PC}$ = 1.5 Hz, 1 C, C(4), 23.6, $^3J_{PC}$ = 9.5 Hz, 1 C, CH_3, 21.0, $^3J_{PC}$ = 2.9 Hz, 1 C, CH_3, 48.9, $^{2or3}J_{PC}$ = 4.4 Hz, 1 C, C(Mu), 42.1, 1 C, C(Mu)). The chromatographically faster isomer **3b** was obtained in approximately 40% yield (mp 67.0–71.0 °C; ^1H NMR ($CDCl_3$) δ 1.33 dd, $^3J_{HH}$ = 6.2, $^4J_{PH}$ = 2.4 Hz, 3 H, $OCCH_3$, 1.35 d, $^3J_{HH}$ = 6.3 Hz, 3 H, $NCCH_3$, 1.64–1.86 m, 2 H, CCH_2C, 2.83–3.04 m, 1 H, NH, 3.12–3.78 m, 9 H, $ClCH_2CH_2$, $NCHCH_3$, .4.45–4.96 m, 1 H, OCH; ^{31}P NMR ($CDCl_3$) δ 10.9; ^{13}C NMR ($CDCl_3$) δ 70.2, $^2J_{PC}$ = 5.4 Hz, 1 C, C(6), 38.5, 1 C, C(5), 46.7, 1 C, C(4), 24.0, 1 C, CH_3, 22.4, $^3J_{PC}$ = 7.8 Hz, 1 C, CH_3, 49.2, $^{2or3}J_{PC}$ = 4.4 Hz, 1 C, C(Mu), 42.3, 1 C, C(Mu)).

Results and Discussion

Synthesis of 2a,b and 3a,b. The *meso*- and *dl*-2,4-pentanediols in Scheme I have been identified by several groups of workers.[17] Interestingly the ratio **5a**/**5b** always exceeded the meso/*dl* diol ratio, and the former ratio was especially larger when workup of the reaction mixture was delayed. It seems likely that acid catalyzes the isomerization of **5b** to the more thermodynamically stable **5a** wherein the methyl groups occupy the sterically preferred equatorial positions. Stereochemical assignments for **5a,b** were arrived at from their NMR spectra. The ^1H NMR parameters for **5a** determined by means of the computer program ITRCAL[18] are quite consistent with a rigid, chair-like conformer with equatorial methyl groups.[19] The chemical shift equivalence of the protons on C(4) and C(6) as well as on C(5) in **5b** (as indicated by the presence of a triplet for the latter protons, a doublet for the C(4) and C(6) methyl protons, and a quartet of triplets for the C(4) and C(6) methine protons[20] in the ^1H NMR spectrum) indicates rapid ring reversal for this molecule on the NMR time scale.

The conversion of other 1,3-diols to amino alcohols via S_N2 attack of ammonia at carbon under the experimental conditions employed here has been proposed earlier.[15] The erythro and threo configurational assignments of the amino alcohols **6b** and **6a** have been made by two groups of investigators. One group[14a] compared relative reaction rates, extent of hydrogen bonding, and pK_a' values for **6a** and **6b** to those of the 1,3-diphenylaminopropanol diastereomers whose configurations had been established by others.[14a] The reported boiling points, although very close to one another, are opposite in order to that which we have found for the two diastereomers, suggesting a conflict in assignment. The other group[14b] deduced from the infrared spectra of **6a** and **6b** that these amino alcohols are intramolecularly hydrogen bonded in the form of cyclohexane-like rings and used the relationship between $^3J_{HCCH}$ coupling and dihedral angle to assign configurations. Insufficient information in this report[14b] prevents us from deciding whether our assignments are the same. Nevertheless, using both **5a** and a mixture of **5a** and **5b**, we find the conversion to the amino alcohols to be stereospecific and, as we show shortly, the stereochemistries of the phosphorinanes **2a,b** and **3a,b** are consistent with inversion at carbon.[15]

Scheme I



As expected, formation of the phosphorinanes from each of the amino alcohols and $MuP(O)Cl_2$ is stereospecific insofar as the relationship of the methyl groups is concerned. Thus the protons of both methyl groups couple to phosphorus in **2a** and **2b** whereas this is the case for only one methyl group in **3a** and **3b.** Extensive investigations of analogous 1,3,2-dioxaphosphorinanes have shown that the protons of equatorial methyl groups on C(4) and C(6) of a chair-like ring couple to phosphorus (1.5–3 Hz) whereas no such coupling is detected when these groups are axial.[21] Furthermore, decoupling the methyl and methylene protons from the OCH methine proton revealed that $^3J_{PH}$ = 1.8 ± 0.2 and 2.5 ± 0.02 Hz for **2a** and **2b,** respectively. Such couplings are characteristic of axially oriented protons.[22] The most likely point wherein inversion at carbon occurs in Scheme I is then the ammonolysis step. Inversion at carbon in the last step of Scheme I is highly unlikely since both alcohols and amines are well-known to attack phosphorus.[23]

Phosphorus Stereochemistry in 2a and 2b. The IR spectra of **2a** and **2b** in the N–H stretching region (Table I) each reveal a higher frequency sharp band and a broader one at lower

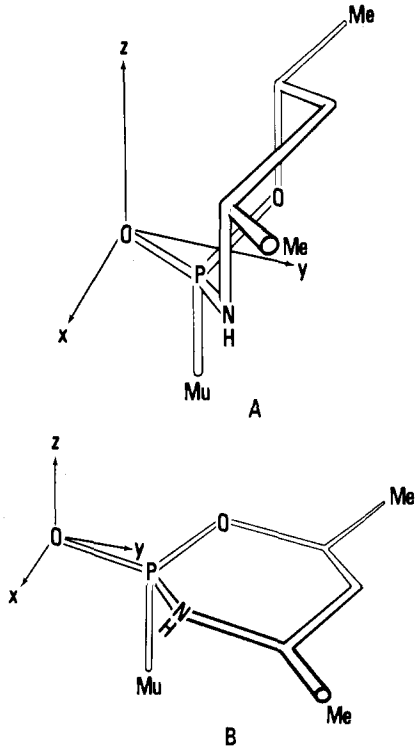


Figure 1. Coordinate system for LIS studies of **2a** (A) and **2b** (B).

Table I. N-H Stretching Region[a,b]

	ν(NH), cm^{-1}		
	free (±3 cm^{-1})		H bound (±10 cm^{-1})
2a		3372 (10)	3170 (4)
2b	3411 (10)		3182 (15)
3a	3411 (7)	3372 (10)	3164 (8)
3b	*c*	3379 (10)	3197 (4)
1	3425 (4)	3375 (10)	3200 (5)

[a] 10^{-3} M concentrations in a cell of 3.5-mm path length. [b] Values in parentheses represent relative peak transmittances. [c] A weak shoulder on the high-frequency side of the 3379-cm^{-1} band is present but its position is very uncertain.

frequency which are reasonably assigned to free and hydrogen-bonded N-H stretching modes, respectively.[24] Only one free N-H band is exhibited by **2a** and **2b** and the frequencies differ significantly. This along with the ^{1}H NMR evidence cited earlier suggests that each of these isomers exists in a rigid configuration. The appearance of these spectra contrasts with those of the other compounds in Table I wherein two free N-H bands are evident, which are indicative of two conformers. It can be concluded then that the isomerism in **2a** and **2b** stems from opposite stereochemistries of phosphorus and that, at least at 10^{-3} M concentrations in CCl_4, there is little if any second conformer for either isomer.

Evidence for a tentative assignment of the phosphorus stereochemistries as shown in **2a** and **2b** in Scheme I is provided by their ^{31}P and ^{1}H(4) and ^{1}H(6) chemical shift relationships. Recent NMR studies of isomeric 2-oxo-2-R-*cis*-4,6-dimethyl-1,3,2-dioxaphosphorinanes have shown that, in general, δ ^{31}P is further downfield for the isomer possessing an equatorial R group.[25] Similarly, an equatorial R group tends to cause lower field chemical shifts for axial methinyl H(4) and H(6) in 1,3,2-dioxaphosphorinanes.[26]

To test these tentative stereochemical assignments, lanthanide-induced shift (LIS) studies on **2a** and **2b** were carried out and the results (Table II) analyzed using the computer program PDIGM.[27] In addition to the assumptions used in solving for a configuration of protons using this program,[28] it was assumed that the structural parameters obtained from the X-ray diffraction study of **1**·H_2O[2b] are valid for these molecules.[29] The program moves the europium relative to the substrate configuration using the axis system in Figure 1; it then compares the observed LIS values (Table II) with those calculated after a two-parameter scaling procedure and then it calculates an *R* factor for each europium position. Conformations A and B in Figure 1 are strongly suggested for **2a** and **2b**, respectively, by the *R* factors in Table III. The lower *R* factors of 5.2 and 10.5% are significantly different from those of the opposite conformation considered for each case at minimum confidence levels of 97.5 and 75%, respectively.[27b] The lower confidence level for **2b** may arise from slight changes in the structural parameters to be expected upon changing from A to B.[30] Interestingly both minimum *R* factors for **2a** and **2b** correspond to a europium position which is offset from the O=P bond axis toward the ring nitrogen, which is suggestive of some degree of chelation of the LIS reagent by these Lewis basic sites.

Table II. Shift (ppm) per Mole Ratio of Eu(dpm)$_3$:Substrate[a]

proton(s)	**2a**	**2b**	**1**
$NCCH_3$[b]	0.48	0.43	
$OCCH_3$[b]	−2.68	−4.23	
CHH_{eq}	−1.45	−3.17	−2.21
CHH_{ax}	−4.24	−6.14	−4.35
NCH	0.34	−4.3	−2.11
NCH			−1.94
Mu[c]	−4.79	−4.19	−4.29
Mu	−7.34	−6.89	−7.34
NH	−4.48	−4.31	−7.26
OCH	−7.72	−6.25	−8.17
OCH			−5.10

[a] The plots of δ ^{1}H for each proton (or group of chemically equivalent protons) vs. the mole ratio of Eu(dpm)$_3$:substrate are nearly linear to a mole ratio of about 0.7.[28] Above this ratio, both positive and negative deviations from linearity occur. Solubility limitations prevented going above a 0.9 mole ratio. Eu(dpm)$_3$ was chosen rather than Eu(fod)$_3$ (even though the latter has greater solubility in CCl_4) because Eu(fod)$_3$ is considerably more acidic and is therefore likely to form complexes with more than one substrate molecule per metal moiety (A. F. Cockerill, G. L. O. Davies, R. C. Harden, and D. M. Rackham, *Chem. Rev.*, **73**, 553 (1973); A.J. Dale, *Acta Chem. Scand., Ser. B*, **30**, 255 (1976)). Positive values denote upfield shifts with added LIS reagent. [b] Assignment was based on the computer analysis of the results. See text. [c] Resonance is split into two halves symmetrical about the center.

Our conclusions regarding the stereochemistries of **2a** and **2b** bear upon the controversy surrounding the conformational properties of **4a** and **4b**.[9,10] One group of investigators assumed the mustard group to be equatorial in both isomers and deduced that **4a** possesses an equatorial C(4) methyl from a comparison of the ^{13}C NMR parameters among **1**, **4a**, and **4b**.[9] The other workers[10] concluded from ^{31}P NMR and IR experiments that the greatly dominant conformer of **4a** contains both the mustard and the methyl group in an equatorial location while **4b** is a conformer mixture with the axial mustard-equatorial methyl conformer dominating in the solvents used. Since the chromatographic and ^{31}P NMR spectroscopic properties of **4a** and **4b** parallel those of **2a** and **2b**, respectively, we favor the assignments of the latter workers.[10] It should be noted that, because of the rigidity imposed by two cis methyl groups, **2a** and **2b** are more representative analogues of the extreme chair conformations of **1** than are **4a** and **4b**.

Solution Stereochemistry of Cyclophosphamide. The positions and intensities of the two free N-H stretching bands in

Table III. Computer Analysis of ^{1}H NMR Spectra of **1**, **2a**, and **2b** Using PDIGM[a]

compd	config[b]	R,[c] %	d_{min},[d] Å	ρ_{min},[e] deg	ϕ_{min},[f] deg
	A	2.2[g]	2.7	60	235
1	B	17.4	2.2	80	300
	A	5.2	1.9	70	240
2a	B	29.0	2.5	110	315
	A	19.4	3.7	80	192
2b	B	10.5	2.0	90	303

[a] Reference 27. [b] Trial configuration in Figure 1. [c] $R = [\Sigma(\text{obsd} - \text{calcd})^2/\Sigma\text{obsd}^2]^{1/2}$ where obsd refers to the LIS values in Table II. [d] d is the distance of the europium to the phosphoryl oxygen. This distance was varied at least from 1.6 to 3.5 Å in increments of 0.1 Å for all structures. [e] The anglo ρ is measured as positive down from the $+z$ axis in the xz plane of Figure 1. This angle was varied from 0 to 180° in increments of 10°. [f] The angle ϕ is measured as positive in a clockwise rotation as viewed down the z axis from the $+x$ axis in the xy plane. This angle was varied from 0 to 360° in increments of $360/\rho$. [g] Three minima with R = 2.25, 3.26, and 3.32% occur at d, ρ values of 2.7, 60, 235; 2.1, 70, 245; and 3.3, 50, 222, respectively.

Table IV. ^{31}P and $^3J_{POCH}$ Values

compd	$^3J_{POCH_A}$, Hz	$^3J_{POCH_B}$, Hz	^{31}P (±0.1 ppm)	solvent
	17.7 ± 0.7[a]	4.7 ± 0.7[a]	12.5	$CDCl_3$
1	13.5 ± 1.5[b]	9.5 ± 1.5[b]	15.3	D_2O
	20 ± 1	<2		*c*
2a		1.8 ± 0.2	12.9	$CDCl_3$
			15.8	D_2O
2b		2.5 ± 0.2	10.2	$CDCl_3$
			13.6	D_2O
3a	15.3 ± 0.2		10.2	$CDCl_3$
3b		2.4 ± 0.2	10.9	$CDCl_3$

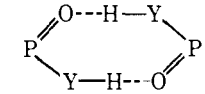
[a] $^3J_{POCH_A} + {}^3J_{POCH_B} = 22.4 \pm 0.4$ Hz. [b] $^3J_{POCH_A} + {}^3J_{POCH_B} = 23.0 \pm 0.5$ Hz. [c] CCl_4 solution with 0.8 mole ratio of Eu(dpm)$_3$:**1** and $CDCl_3$ added for spectrometer locking.

the IR spectrum of **1** (Table I) indicate that two conformers are present with the major one resembling the configuration of **2a.** In contrast to slow conformer interconversion on the IR time scale, however, this process is rapid in the NMR experiment. From the simplification of several multiplets and the broadening of other ^{1}H NMR resonances upon cooling **1** in $CDCl_3$, rapid conformer interconversion has been postulated.[8] Upon decoupling the C(5) protons from the OCH_2 protons of **1** in $CDCl_3$ and analyzing the resulting AB part of the ABX spectrum (X = ^{31}P) we find that $^3J_{POCH}$ = 17.7 and 4.7 Hz for the two protons (Table IV). From the dependence of this coupling on the POCH dihedral angle[22] and the significantly smaller values of $^3J_{POCH}$ for **2a** and **2b** it can be concluded that the values of $^3J_{POCH_A}$ and $^3J_{POCH_B}$ in Table IV for **1** stem from weighted averages of conformers which are likely to be in the chair form and which differ in configuration at phosphorus. Assuming that the methyl groups in **2a** and **2b** do not significantly influence δ ^{31}P, and that these isomers are representative of the extreme chair conformers of **1**, as has been successfully assumed for related ring systems,[21] then the ^{31}P chemical shift of **1** indicates a ratio **1a:1b** of about 3 in D_2O. The higher ratio 6 suggested by this parameter when measured in $CDCl_3$ is corroborated by the more disparate $^3J_{POCH_A}$ and $^3J_{POCH_B}$ values in this solvent (Table IV). If one assumes that $^3J_{POCH_{ax}}$ is 2.2 Hz (i.e., the average $^3J_{POCH}$ for **2a** and **2b**) and $^3J_{POCH_{eq}} = {}^3J_{POCH_A} + {}^3J_{POCH_B} - {}^3J_{POCH_{ax}} = 20.2$ Hz in $CDCl_3$, then the observed values of $^3J_{POCH_A}$ and $^3J_{POCH_B}$ for **1** are also consistent with a chair conformer ratio of about 6:1. The greatest disparity in the values for these couplings occurs when **1** is dissolved in CCl_4 containing Eu(dpm)$_3$. This result is in harmony with the high confidence level of 99% associated with the R value for conformation **1a** in the presence of LIS reagent (Table III). The favoring of this conformer in the presence of a complexing agent is reasonable in view of the greater basicity of an axial phosphoryl oxygen compared with an equatorially disposed oxygen.[25] Moreover, the phosphoryl oxygen and ring nitrogen lone electron pairs are geometrically better oriented for chelation with europium in **1a** than in **1b.** It is likely then that the conformational equilibrium of **1** at the point where the drug is oxidized by liver microsomes[1] will depend highly on the polarity and Lewis acidity of the local environment.

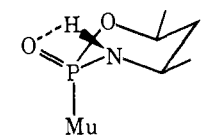
Stereochemistry of 3a and 3b. The relative intensities of the free N-H stretching modes for **3a** and **3b** in Table I reveal comparable concentrations of two conformers for **3a** but a highly favored conformer for **3b.** The favored conformer in each case possesses a free N-H frequency which is close to that displayed by **2a,** which would be consistent with postulating that all three species have the same configuration at phosphorus (i.e., equatorial mustard, axial phosphoryl oxygen). However, δ^{31}P for **3a** and **3b** (Table IV) are considerably closer to that of **2b.** Although distortion from chair-form conformers arising from the trans disposition of the ring methyl groups may be responsible for the inconsistency of the IR and ^{31}P spectral data for **3a** and **3b,** it is not possible to decide unambiguously which, if either, set of spectral results can be relied upon for the phosphorus stereochemical assignment in the dominant conformers.

Partial analysis of the ^{1}H NMR spectra does show how the dominant conformers of **3a** and **3b** differ, however. The $^3J_{POCH}$ values for these compounds are quite different, with the value for **3b** being quite close to those of **2a** and **2b.** These results permit the conclusion that the dominant conformer of **3b** contains at least an approximately equatorial OCMe methyl group and hence an approximately axial NCMe methyl group. The value of $^3J_{POCH}$ for **3a** is lower than expected for an equatorial OCH proton in this system and this supports the IR evidence for the presence of more than one conformer. Further support for this conclusion is derived from the value of $^3J_{HC(6)C(5)H} + {}^3J_{HC(6)C(5)H'}$ (9.2 Hz) for **3a,** which is larger than expected for an equatorial OC(6)H coupling to axial and equatorial C(5) protons and less than expected for an axial OC(6)H coupling.[19]

Effects of Hydrogen Bonding. At 10^{-1} M in CCl_4, the bound N-H stretching band is much more intense than the free band(s) for **1**, **2a,b,** and **3a,b.** Except for **2b**, the opposite is true at 10^{-3} M (Table I), which is consonant with the idea that the hydrogen bonding at higher concentrations is largely intermolecular. Dimer formation of the type shown below is well-known where Y = O.[23] The persistence of a relatively intense



bound N-H band in **2b** may be attributed to the retention of the above hydrogen-bound dimers which have been proposed to account for the IR characteristics of 2-oxo-1,3,2-diazaphosphorinanes.[24] It is likely that the four-membered ring shown below also contributes to the bound N-H band in **2b**



2b

because of the closer proximity of the oxygen and the proton when the oxygen is equatorial than when the oxygen is axial as in **2a.** Evidence for such four-membered rings has been accumulated in IR studies of phosphoramidates.[31] The above arguments support an equatorial mustard group in each of the dominant conformers of **3a** and **3b** as do the free N-H frequencies discussed earlier.

In the environment in which cyclophosphamide metabolizes, there are undoubtedly many sites for hydrogen bonding which would hinder dimer formation. This is substantiated in the solid state by the observations that in **1**·H_2O molecules of **1** are hydrogen bound to water via the phosphoryl oxygen and the NH hydrogen[2a] whereas in the structure of anhydrous (+)-**1**[5] trimeric units of **1** occur in the unit cell. Furthermore, hydrogen bonding, like LIS complexation, is expected to enhance the stability of the **1a** conformer owing to the greater hydrogen-bonding capability of an axial oxygen.[25]

The phosphoryl group stretching region (1200–1260 cm^{-1}) for **2a** and **2b** shows three bands and **1** shows five in CCl_4. Hydrogen bonding is undoubtedly largely responsible for the complexity of this spectral region and therefore assignment of the phosphoryl group stereochemistry as has been accomplished in related simpler systems[21] is not possible.[32]

Dipole moment measurements in rigid isomeric 1-R-2-oxo-4,6-dimethyl-1,3,2-dioxaphosphorinanes have proven very successful in differentiating stereochemistries at phosphorus and also in elucidating the conformational bias in the labile analogues lacking the 4,6-dimethyl substituents.[21] The rigid model isomers **2a** and **2b,** which are representative of the chair conformations of **1**, however, differ by only 0.42 D in benzene in the 1.0×10^{-3} to 3.5×10^{-3} mole fraction range. Moreover, plots of dielectric constant vs. solute mole fraction exhibit marked curvature above ca. 3.5×10^{-3} mole fraction in the direction of smaller dipole moment. It is probable that additional conformational possibilities from the flexible mustard groups, the lower symmetry of these ring systems compared to dioxaphosphorinanes, and the opportunities for hydrogen bonding combine to reduce the difference in molecular moments due to stereochemical isomerism at phosphorus in **2a** and **2b,** thereby rendering this technique ineffective in clarifying the conformational bias of **1**.

Conclusions

Using **2a** and **2b** as model compounds for solution spectroscopic properties of conformers **1a** and **1b** of cyclophosphamide, it becomes reasonable to conclude from a comparison of such properties that the dominant conformer of **1** in solution is **1a.** It is apparent that **1a** is more stable in the presence of $Eu(dpm)_3$ in CCl_4 and less stable (though still more so than **1b**) in $CHCl_3$ and H_2O. Factors which may be responsible for variations in conformer ratio include differences in conformer phosphoryl oxygen basicities, chelating abilities toward $Eu(dpm)_3$, and stabilization by hydrogen-bonding solvents.

Although it is generally the case that ring substitution decreases anticancer activity compared to **1**,[9,33] it is interesting to note that **2a,** which possesses the configuration matching the dominant conformation of **1** (i.e., **1a**), shows preliminary activity against KB tumor cell cultures whereas **2b** does not. This result is suggestive, albeit weakly, that conformation **1a** is more active than **1b.**

Acknowledgment. J.G.V. thanks the National Cancer Institute of the NIH for support of this research in the form of a grant and for screening data.

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(28) The assumptions follow:[27] (a) The lanthanide–substrate complex can be described by a single set of coordinates. The IR and NMR results for **2a** and **2b** discussed previously support the use of this assumption for all but the mustard protons which are conformationally mobile in solution. These protons were therefore not included in the analysis. (b) The lanthanide atom is approximately axially symmetric with respect to its magnetic properties. (c) The principal magnetic axis of the lanthanide moiety passes through the Lewis base site of complexation. (d) The LIS is effectively pseudocontact in origin. Recently reported studies are supportive of this assumption (T. A. Gerken and W. M. Ritchey, *J. Magn. Reson.,* **24,** 155 (1976)).
(29) The coordinates of the ring protons in **2a** and **2b** were derived from the molecular structure of 1·H_2O[2b] assuming C–H and N–H distances of 1.05 and 1.02 Å respectively, HCH and HCC angles of 109.54°, and HNC and HNP angles of 119.25°. An average methyl proton was placed 1.88 Å from the C(4) and C(6) carbons along the C–CH_3 bond. For **2b,** the mustard and phosphoryl oxygen groups were interchanged so as to retain as closely as possible the angles of 1·H_2O. The atomic coordinates and requisite dihedral angles for PDIGM were calculated with the aid of programs ATCOR with subroutine RELOC3 (D. Applequist, Department of Biochemistry, Iowa State University, as obtained from J. E. Nordlander, Department of Chemistry, Case Western Reserve University) and DAPT (R. A. Jacobson, Department of Chemistry, Iowa State University).
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(32) Others[10] assigned a band at 1218 cm^{-1} to $\nu P{=}O_{ax}$ in **4a** and an absorption at 1231 cm^{-1} to $\nu P{=}O_{eq}$ in **4b**. Correlation of these bands with the most intense band in the spectra of **1, 2a, 2b, 3a,** and **3b** leads to stereochemical assignments consistent with those achieved by the other techniques discussed in the present paper except for **1**. Thus, except for more simple systems, interpretation of this region of the infrared in terms of stereochemical implications is fraught with risk.
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